C/EBPα initiates primitive myelopoiesis in pluripotent embryonic cells

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Abbreviations:
aVBI: anterior ventral blood island
pVBI: posterior ventral blood island
CHX: cycloheximide
MO: morpholino oligonucleotide
Abstract

The molecular mechanisms that underlie the development of primitive myeloid cells in vertebrate embryos are not well understood. Here we characterize the role of *cebpa* during primitive myeloid cell development in *Xenopus*. We show that *cebpa* is one of the first known hematopoietic genes expressed in the embryo. Loss and gain-of-function studies show that it is both necessary and sufficient for the development of functional myeloid cells. In addition, we show that *cebpa* misexpression leads to the precocious induction of myeloid cell markers in pluripotent prospective ectodermal cells, without the cells transitioning through a general mesodermal state. Finally we use live imaging to show that *cebpa* expressing cells exhibit many attributes of terminally differentiated myeloid cells, such as highly active migratory behavior, the ability to quickly and efficiently migrate toward wounds and phagocytose bacteria, and the ability to enter the circulation. Thus C/EPBα is the first known single factor capable of initiating an entire myelopoiesis pathway in pluripotent cells in the embryo.
Introduction

Hematopoiesis occurs in two distinct phases during development.\textsuperscript{1-4} The first wave, also known as primitive hematopoiesis, often occurs in extraembryonic hemogenic sites and provides the embryos with a transient population of blood cells. The second wave gives rise to blood progenitors, which persist into late development and adulthood in successive hemogenic sites, and is therefore called definitive hematopoiesis. While much is known about the molecular and cellular pathways responsible for definitive hematopoiesis, relatively little is known about the pathways responsible for the specification of the primitive blood lineages.

In the past decade, aquatic vertebrate species have emerged as powerful model organisms for the investigation of primitive and definitive hematopoiesis.\textsuperscript{5-8} Favored aquatic model organisms for hematopoiesis include the teleost fish, zebrafish, and the amphibian, \textit{Xenopus laevis}, and its diploid relative, \textit{Xenopus tropicalis}.\textsuperscript{9,10} Fish and frog embryos can be produced in large numbers and they develop externally, allowing the visualization of the development and behavior of blood lineages \textit{in vivo}. Since the embryos are large, embryological manipulations, such as tissue transplantations, can be performed with relative ease. In addition, the molecular and genetic basis of hematopoiesis in these organisms is largely conserved with those of mammals.\textsuperscript{5-8}

Studies in zebrafish have shown that primitive hematopoiesis consists of two separate events: primitive myelopoiesis and primitive erythropoiesis, which take place in two distinct hematopoietic compartments.\textsuperscript{11,12} Similarly in \textit{Xenopus}, the primitive myeloid-forming compartment is located in the anterior ventral blood islands (aVBI), which are derived from dorsal/anterior gastrula mesoderm, while primitive erythropoiesis occurs primarily in the
posterior ventral blood islands (pVBI), which are derived from ventral/posterior gastrula
mesoderm.\textsuperscript{1,13-15}

We recently showed that primitive myeloid cells are the first blood cells to differentiate
and become functional in the \textit{Xenopus} embryo.\textsuperscript{15} We also showed that these early differentiating
myeloid cells are quickly and efficiently recruited to embryonic wounds before a functional
vasculature is established.\textsuperscript{15} We are particularly interested in investigating the role of these
primitive myeloid cells during tissue formation and repair in the embryo. Before addressing
these questions, however, we sought to learn more about how these cells are specified. Given
that many of the pathways responsible for definitive hematopoiesis are also used during
primitive hematopoiesis, we have endeavored to address the role of C/EBP\textgreek{a}, a member of
CCAAT/enhancer binding protein family, during primitive myelopoiesis.

C/EPB\textgreek{a} has long been recognized as an important factor during definitive myelopoiesis,
where it is a key factor in driving common myeloid progenitors (CMPs) to differentiate into
granulocyte-monocyte progenitors (GMPs).\textsuperscript{16-18} Subsequently, C/EBP\textgreek{a} has a recurrent role in
driving myeloid progenitor cells toward terminal differentiation.\textsuperscript{16,17} In addition, mutations in
C/EBP\textgreek{a} are commonly found in patients with acute myeloid leukemias.\textsuperscript{19,20} In zebrafish,
overexpression of a dominant interfering \textit{cebpa} construct promotes primitive erythropoiesis,
without a discernable effect on granulopoiesis.\textsuperscript{21}

Here we characterize the function of \textit{cebpa} during primitive myeloid cell development in
\textit{Xenopus}. We found that \textit{cebpa} is one of the first known hematopoietic genes expressed in
\textit{Xenopus} embryos. Furthermore, gain and loss-of-function studies show that \textit{cebpa} is both
necessary and sufficient for the differentiation of functional myeloid cells in the embryo. Finally
we show that *cebpa* can induce naïve embryonic cells along the myelopoiesis pathway, resulting in precocious differentiation of functional myeloid cells.
Materials and Methods

Isolation of cebpa and Construct Design

A full-length clone encoding *X. tropicalis cebpa* (TEgg058d02) was identified in the Gurdon Institute *Xenopus tropicalis* Full Length Database (http://informatics.gurdon.cam.ac.uk/online/xt-fl-db.html).\(^2\) HA-cebpa and pCS2 cebpa were generated by PCR from TEgg058d02, using the following primers: 5' GGA TCC ATG GAT CAA GCC AAC TTC 3' and 5' GAA TTC TTA TGC ACA GTT GCC C 3'. After digestion with EcoRI and BamHI, the PCR product was subcloned into both pFTX11 and pCS2 vector, and verified by sequencing.

Whole-mount in situ hybridizations

Whole-mount in situ hybridizations were performed according to established protocols.\(^2\) Probe synthesis for cebpa, spiB, spiL, scl, mpo, fliL, lcp, mmp7 and globin was done according to Costa et al. (2008).\(^1\) In addition, probe synthesis for csf1r (colony stimulating factor 1 receptor) was done using IMAGE clone:7015756 (EcoRV, T7) and for gsc using clone TNeu077f20 (EcoRI, T7). For X-gal staining, fixed embryos were incubated in a solution containing 4 mM potassium hexacyanoferrate (III), 4 mM potassium hexacyanoferrate (II), 2 mM MgCl\(_2\) and 0.4 mg/ml X-gal at 37°C for 1 hour. The extent of migration was scored according to Tomlinson, *et al.* (2008).\(^2\) Image acquisition was performed on a Leica MZAPO stereomicroscope using Northern Eclipse software (Empix Imaging Inc., Mississauga, ON).
**RNA synthesis and Embryo Microinjections**

Capped mRNA was synthesized using the mMessage Machine kit according to the manufacturer’s directions (Ambion, *cebpa*: SP6; HA-*cebpa*: T7). Embryos were cultured and injected with RNAs according to established protocols. Developmental staging was done according to Nieuwkoop and Faber (1994). The same quantity of eGFP mRNA was injected as a control. Frog embryo experiments are covered by a Project License issued by the Home Office in the United Kingdom.

**Morpholino Design and Microinjections**

*cebpa* is an intronless gene and can be knocked down by injecting translational blocking morpholino oligonucleotides overlapping the start coding of the gene. MOs were purchased from Gene Tools LLC. *X. tropicalis* embryos were injected with 10ng total MOs before first cleavage, and generally a fluorescent control MO (Li std; Gene Tools) was injected as control. MO sequences are listed as follows:

- *cebpa*ATG 5’-CTCGTAGAAGTTGGCTTGATCCATG-3’
- standard control MO 5’-CCTCTTACCTCAGTTACAATTTATA-3’

**Immunobloting**

Synthetic HA-*cebpa* or *cebpa* mRNA (300 pg) was injected into *Xenopus laevis* embryos at 1-cell-stage, with or without 10ng of *cebpa*ATG MO. Embryos were harvested at stage 12 and homogenized in 1× RIPA lysis buffer with the addition of Complete protease inhibitor cocktail (Roche). The equivalent of one embryo was loaded and run on a 15% SDS-PAGE gel. After
electrophoresis, proteins were transferred to PDVF membrane (Millipore) and probed with 1:1000 goat anti-CEBPA polyclonal antibody (C/EBPα (C-18):sc-9314, Santa Cruz) or 1:1000 mouse anti-HA HRP (clone 3F10, 1667475, Roche).

**RNA isolation and Real-time PCR**

Total RNA was extracted from pools of embryos or animal caps using TRIzol reagent according to manufacturer’s instructions (Invitrogen). cDNA was synthesized using BioScript™ reverse transcriptase (Bioline), and PCR reactions were performed using Taq polymerase (Roche) according to established protocols. Primer sequences are shown in the supplementary Table. The data for each sample was normalized relative to the expression level of ribosomal protein L8 (rpl8) and calculated by the 2^{-ΔΔCt} method. Real-time RT-PCR analysis was performed using a Chromo4™ Real-Time PCR Detector (Bio-Rad).

**Animal cap assay and mesodermal inhibition**

*X. laevis* embryos were injected into the animal pole at the 2-cell stage and animal cap explants were excised at stage 8. Caps were then incubated and cultured in 75% NAM containing 0.2% BSA with or without 10μg/ml cycloheximide (Sigma) until the required stage. In order to block mesoderm formation, both an FGFR inhibitor, 20μM SU5402, and a Nodal signaling inhibitor, 200μM SB505124, were added into the medium from 2-cell stage. Same volume of DMSO was added as control.

**Animal cap transplantation and live imaging**

HA-cebpa mRNA (200 pg) was co-injected with eGFP mRNA (200 pg) into the animal pole of
Xenopus laevis embryos at the one cell stage. As a control, only eGFP mRNA was injected. Animal caps were harvested using an eyebrow knife at stage 8. Meanwhile synchronized unlabelled host embryos were prepared by removing a similar size piece of animal cap. The labelled animal caps were placed on the host embryos, such that the margins between the transplant and host embryo matched. Transplants were allowed to heal in 0.4 X MMR for 1 hour, and then the chimeric embryos were transferred to 0.1 X MMR for long-term culture. Wounds were created using forceps. A suspension of mCherry positive bacteria in 1xPBS was injected for the phagocytosis experiments. Time-lapse fluorescent image acquisition was done using a Leica MZFLIII stereoscope and Northern Eclipse software (Empix Imaging Inc.). Confocal imaging was done using an Olympus IX81 FluoView FV1000 microscope (Olympus Inc., Tokyo, Japan).
Results

Characterization of cebpα expression in Xenopus tropicalis embryos

To better understand the molecular mechanisms of primitive hematopoiesis in Xenopus tropicalis, we identified a panel of hematopoietic genes from our Xenopus tropicalis EST database and analyzed their embryonic expression by whole-mount in situ hybridization (Fig. 1). While cebpα expression could be detected robustly at the mid gastrula stage (Fig. 1, Stage 12; Fig. S1A-C, in supplementary material), the timing of expression of all the other early hematopoietic markers tested could not be seen until the neurula stages (stage 15; Fig. 1). In the gastrula stage, the region, which expressed cebpα, corresponded to the anterior mesendoderm, marked by the expression of gsc (Fig S1D-F, in supplementary material). This region of the gastrula is fated to give rise to the aVBI. During the neurula stages, cebpα expression was restricted to the aVBI (Fig. 1, stages 15 and 18). At the early to mid-tailbud stages (Fig. 1, stage 23 and 26, respectively), cebpα expression showed a punctate pattern, consistent with the initiation of migration of primitive myeloid cells throughout the embryo. The spatial expression pattern of cebpα during the neurula stage and tailbud stages resembled that seen by several primitive myeloid markers, including spib, mpo, mmp7, lcp (l-plastin), csf1r (colony stimulating factor 1 receptor) and spi1 (also known as PU.1), suggesting that cebpα is expressed in primitive myeloid cells (Fig. 1). However, the whole-mount in situ hybridization data showed that cebpα was detectable considerably earlier than any other blood marker, including scl and flil, early hemangioblast markers or spib, an early marker of primitive myeloid cells (Fig. 1). Consistent with this result, temporal RT-PCR analysis also showed that cebpα was expressed prior to all tested myeloid genes. These data suggested that cebpα may function very early in
the hematopoietic gene regulatory cascade.

**C/EBPα depletion down-regulates the expression of myeloid markers and inhibits terminal myeloid differentiation**

The timing and pattern of expression of *cebpa* encouraged us to investigate its function during primitive hematopoiesis. We first determined the effect of knocking down *cebpa* function in embryos using antisense morpholino oligonucleotides (MOs). Given that the coding sequence of *cebpa* is not disrupted by introns, we designed a MO complementary to the start codon of *cebpa*, hereafter named *cebpa*ATG. In order to test whether the *cebpa*ATG MO could efficiently inhibit translation of *cebpa*, we co-injected *in vitro* transcribed *cebpa* mRNA (300 pg) with the *cebpa*ATG MO (10 ng) into embryos and performed immunoblotting with a commercially available C/EBPα antibody (Fig. S2, in supplementary material). We found that co-injection of the *cebpa*ATG MO completely inhibited the translation of *cebpa*. However, by adding an HA tag upstream of the start codon of *cebpa*, the *cebpa*ATG MO was unable to inhibit translation. Thus we concluded that the *cebpa*ATG MO was efficient in inhibiting the translation of wild type *cebpa*, but not the HA-C/EBPα construct.

We then tested the effect of knocking down *cebpa* in *Xenopus tropicalis* embryos by assaying the expression of a large panel of primitive myeloid markers, including *mpo*, *lcp*, *mmp7*, *spi1*, *mmp7*, *spib* and *cebpa* (Fig. 2). In control MO injected mid-tailbud staged embryos (stage 23), cells expressing these markers have begun to migrate away from the aVBI and spread throughout the embryo (Fig. 2A, C, E, G, I, K). However, the migration of these cells in *cebpa*ATG MO injected embryos was severely inhibited, suggesting that the cells were not differentiating properly into functional myeloid cells (Fig. 2B, D, F, H, J, L). In addition,
terminal differentiation markers, such as mpo, lcp and mmp7 were significantly reduced or absent in the cebpaATG MO injected embryos (Fig. 2B, D, F). To quantify this effect, we performed real-time PCR analysis on mpo, lcp, mmp7, spi1 and spib (Fig. 3C). With the exception of spib, all of these markers were significantly down-regulated (P<0.05). However, hemangioblast markers, such as scl and fli, as well as the erythroid marker, globin, were not affected by knocking down cebpa (Fig. S3, in supplementary material). Interestingly the expression level of endogenous cebpa was up-regulated in the cebpa morphants (Fig. 2J), suggesting either that the endogenous mRNA is stabilized by the cebpaATG MO, or cebpa is involved in its own down-regulation.

To address the specificity of the cebpaATG knock down, we asked whether the HA-C/EBPα construct, which we previously showed was not affected by the cebpaATG MO, could rescue the knock down effect of the MO, both in terms of migration and gene expression. To determine whether the HA-C/EBPα construct could rescue the migration defect, we injected either the control or experimental cebpaATG MOs at the one cell stage (Fig 3Ai), and then we co-injected 20 pg of HA-cebpa or gfp mRNA with 50pg of β-gal mRNA at the 32-cell stage, into the two C1 blastomeres, which give rise to aVBI (Fig. 3Aii). When the embryos reached the mid-tailbud stage (stage 23), the embryos were fixed and stained with X-gal (Fig. 3Aiii-v). While control injected embryos contained X-gal positive cells covering 40% of the embryo (Fig 3Aiii and 3B), the cebpaATG MO injected embryos contained X-gal positive cells in less than 20% of the embryo (Fig 3Aiv and 3B), which is essentially the size of the aVBI. However, the cebpaATG MO injected embryos rescued with the HA-CEBPα construct contained X-gal stained cells in over 40% of the embryo (Fig. 3Av and 3B), suggesting that the HA-CEBPα
construct was able to fully restore the migratory behaviour of the myeloid cells emanating from the aVBI.

We then determined whether the HA-C/EBPα could rescue the expression of myeloid markers in cebpaATG MO injected embryos. As seen in Fig. 3C, not only were all markers restored in the HA-CEBPα injected embryos, but all the myeloid markers were significantly up-regulated over their normal levels. Indeed, even spib, which was not significantly down-regulated by the cebpaATG MO, was up-regulated by the HA-cebpa mRNA (Fig. 3C v). These data suggested that the cebpa knock down phenotypes were specific, and that overexpression of cebpa mRNA leads to an up-regulation of all myeloid marker genes tested.

**Overexpression of cebpa induces ectopic expression of blood markers in Xenopus tropicalis embryos**

A potential gain-of-function phenotype was suggested by the morpholino rescue experiments, in which primitive myeloid markers were up-regulated by HA-C/EBPα overexpression. To further investigate the ability of C/EBPα to induce primitive hematopoiesis, we ectopically expressed cebpa in Xenopus tropicalis embryos by injecting cebpa mRNA randomly into a single cell at the 8-cell stage. Given that high levels of expression of cebpa can lead to embryonic death (data not shown), we injected low amounts of cebpa mRNA into embryos (10 pg per cell at the 8-cell stage), which resulted in an over 95% survival rate. Under these conditions, C/EBPα caused extensive ectopic expression of several blood markers in a significant percentage of embryos (Fig. 4 and Fig. S4, in supplementary material, spib: 54.2% (n=35); mpo: 36.1% (n=36); mmp7: 19.5% (n=41); lcp: 31.2% (n=32); fli1: 36.8% (n=38); spi1: 18.9% (n=37); scl: 25.7% (n=35)).

The normal expression of all these markers at stage 18 is localized to the aVBI (Fig. 1).
However, in embryos injected with *cebpa* mRNA, ectopic expression was detected in many regions of the embryo, including the neural folds, and lateral and posterior regions of the embryo. Thus, misexpression of *cebpa* can lead to a dramatic expansion in the expression of early hematopoietic and myeloid markers throughout the embryo. One possible cause for this could be that the existing pool of myeloid progenitors undergo over proliferation and premature migration. Alternatively, this expansion of myeloid expressing cells could be the result of an ectopic induction of myelopoiesis in the embryos.

**cebpa induces the expression of blood markers in pluripotent cells**

To address whether C/EBPα could induce ectopic expression of myeloid genes, we performed gain-of-function analyses in pluripotent embryonic cells isolated from early Xenopus embryos. Although normally fated to become epidermis, cells in the animal pole of blastula stage embryos (referred to as animal caps) are competent to differentiate into a broad range of tissue types, including neural, mesodermal or endodermal cell types, given the appropriate signals. We wondered whether C/EBPα could exert an effect on these pluripotent embryonic cells. When *cebpa* was misexpressed in animal caps, all blood markers tested, with the exception of *globin* and *rag1*, were significantly up-regulated (Fig. 5A). Moreover, the induction of these hematopoietic genes occurred precociously, well before they would normally be expressed in the embryo. To further annotate these results, we divided all hematopoietic genes examined into two categories with respect to how quickly they responded to misexpression of *cebpa*. Early up-regulated genes included *spib, lurp* and *fli1*; by the mid gastrula stage (stage 11), these genes had already been highly up-regulated in *cebpa* mRNA injected animal caps (Fig. 5A i, ii, v).
Late up-regulated genes included mpo, mmp7 and scl; these genes were dramatically up-regulated at the neurula stage (stage 15), but much less so at stage 11 (Fig. 5A iii, iv, vi). The erythroid marker, globin, and lymphoid marker, rag1,29 were not activated by cebpα misexpression (Fig. 5A x). Furthermore, neither the early liver marker, forl,30 nor the early lung marker, nkx2.1,31 were upregulated precociously by cebpα (data not shown). Similarly, neither the early mesodermal markers, xbra and gsc, nor the ectodermal marker keratin were up-regulated in cebpα mRNA injected animal caps (Fig. 5A vii-ix), suggesting that cebpα specifically induced the early hematopoietic and myeloid genes.

To address whether some of these genes might be direct transcriptional targets of C/EBPα, we asked which genes could be activated in the absence of new protein synthesis. In Xenopus, zygotic transcription does not normally start until the mid-blastula transition, which occurs at stage 8.32 Therefore, we allowed translation of the cebpα mRNA following injection at the one cell stage until stage 8. We then isolated the animal caps and tested for the activation of the marker with or without the protein synthesis inhibitor cycloheximide (CHX). From this experiment, we found that the early-induced genes, spib and lurp, were activated in the presence of CHX, while the late-induced genes (mpo and mmp7) were not (Fig. 5B). These results suggest that spib and lurp are direct targets of C/EPBα, while mpo and mmp7 are indirect targets of C/EPBα. We next scanned the promoter region of X. tropicalis spib and lurp for putative C/EBP binding sites. From this analysis, we found that the X. tropicalis spib promoter contains a putative C/EBP binding site at position -160 through -171 and the lurp promoter contains another at position -147 through -156 (data not shown). Interestingly, the murine Spib promoter also contains a putative C/EBP binding site in a conserved position.33
C/EBPα does not induce general mesodermal markers in animal caps

Our animal cap data suggests that cebpα overexpression is capable of driving pluripotent embryonic cells along the myelopoiesis pathway without the cells transitioning through an early general mesodermal state. However, given that cebpα induces myeloid markers precociously, we wondered whether the cells were passing through a very transient general mesodermal state, which had been missed in our experiments. To address this possibility, we assayed the expression of the early mesodermal markers xbra, gsc and eomes by real-time PCR from the beginning of zygotic transcription (i.e. stage 8) (Fig. 6A). We isolated animal caps from embryos at stage 8, and we analyzed the expression level of xbra, gsc and eomes at stages 8, 9, 10 and 11. The resulting real-time PCR data showed that cebpα did not induce the expression of the early mesodermal marker genes at any point in animal caps, although these genes could be detected robustly in whole embryos starting from stage 9 (Fig. 6A). In contrast, an early myeloid marker, spib, was robustly induced in the cebpα overexpressing animal caps, starting from stage 9, even though spib expression was not detected in the whole embryos or the control animal caps at any stage between stage 8 through 11.

We next asked whether cebpα could induce myeloid markers under conditions where mesoderm formation is blocked using the FGF receptor and nodal (ALK5) receptor inhibitors, SU5402 and SB505124, respectively. When we treated embryos with both inhibitors from the 2-cell stage, the embryos failed to express xbra and gsc at the gastrula stages (Fig. 6Bi, ii), suggesting that these two inhibitors can completely block the formation of mesoderm. We then asked whether cebpα could induce myeloid markers in the presence of these two inhibitors. We
found that the myeloid genes *spib*, *lurp* and *mmp7* were still induced in *cebpa* overexpressing animal caps at stage 11, even in the presence of both inhibitors (Fig. 6Biii-v). Taken together, these data suggest that *cebpa* can induce primitive myeloid markers without the cells transitioning through an early mesodermal state.

**Ectopic expression of C/EBPα induces an entire myelopoiesis program in pluripotent embryonic cells**

While the real-time PCR data were suggestive that C/EPBα might be initiating the hematopoietic regulatory cascade in the animal cap cells, we wanted to test whether the cells were becoming functional myeloid cells. To answer this question, we injected *gfp* mRNA into the animal pole of embryos with and without *cebpa* mRNA and performed animal cap transplantations into unlabelled host embryos (Fig. 7A). By using live time-lapse fluorescence imaging we were able to monitor the behaviour of the graft-derived cells during later stages of development (Fig. 7B). While control eGFP labelled animal cap cells remained contiguous, underwent epiboly and formed epidermis, eGFP labelled animal cap cells expressing *cebpa* started to migrate away from the graft from the late gastrula stages (see Video S1 in supplementary material). The *cebpa* expressing transplanted cells became migratory nearly a day before the endogenous primitive myeloid cells migrate away from the aVBI, consistent with the precocious up-regulation of blood markers seen in *cebpa* expressing animal cap explants (Fig. 6). While the boundaries between eGFP positive control transplants and the non-fluorescent host cells remained distinct throughout development (Fig. 7B i, ii), the *cebpa* expressing transplanted cells could be found in a spotty pattern throughout the embryo at the
tailbud stages (Fig. 7B, iii, iv). We noted from these transplantation experiments that, while many C/EBPα/eGFP positive cells became migratory, some retained the morphology of epidermal cells, suggesting that not all C/EBPα/eGFP cells become myeloid cells. Later on, migratory cells showed ramified multipolar morphology (Fig. 7B, v), characteristic of terminally differentiated macrophages. In addition, these cells displayed extremely active migratory behaviour (see Video S2, in supplementary material), characteristics of terminally differentiated primitive myeloid cells. Like terminally differentiated myeloid cells, a subset of these cells entered the circulation in the embryos (see Video S3, in supplementary material).

We have previously shown that terminally differentiated primitive myeloid cells can be quickly and efficiently recruited to embryonic wounds. Therefore, we tested whether these animal cap-derived cells expressing C/EBPα and eGFP in chimeric embryos could respond to embryonic wounds. Before wounding, a subset of C/EBPα/eGFP positive cells patrolled the embryo randomly (see Video S4, in supplementary material and Fig. 7C), but after wounding, these cells immediately responded by migrating toward the wound site (see Video S5, in supplementary material and Fig. 7C). In contrast, control eGFP transplanted cells failed to respond to wounds (see Video S6, in supplementary material).

We next asked whether cebpa expressing animal cap cells were capable of phagocytosis. We transplanted animal cap cells expressing either C/EBPα and eGFP or eGFP alone into unlabelled host embryos and allowed these chimeric embryos to develop into the tailbud and tadpole stages. We then injected mCherry expressing E. coli into these chimeric embryos. As shown in supplementary Video S7, the transplanted C/EBPα/eGFP positive cells quickly migrated to the infection site and engulfed the red bacteria. While the C/EBPα/eGFP cells can
be visualized initially in the green channel only, after engulfing the bacteria, migratory myeloid cells became both green and red. Twenty-four hours after infection, the C/EBPα/eGFP positive cells, which have phagocytosed the red fluorescent bacteria, could be seen migrating throughout the embryo. We even detected these green/red myeloid cells re-entering the circulation.

In order to obtain a better resolution of the morphology of the cells, we used time-lapse confocal microscopy. Using this method, we were able to follow the morphology and behaviour of the C/EBPα/eGFP positive cells that had engulfed the red fluorescent bacteria (green/yellow cells in supplementary Video S8), C/EBPα/eGFP positive cells that had not engulfed bacteria (green cells in supplementary Video S8), and the endogenous myeloid cells that had phagocytosed the fluorescent bacteria (red cells in supplementary Video S8). The ramified morphology of the C/EBPα/eGFP positive cells is reminiscent of macrophages grown on epithelial substrates. Analysis of the confocal images in three dimensions, show that the engulfed bacteria were present in intracellular compartments within the cytoplasm of the C/EBPα/eGFP positive macrophages (Fig. 7D). Further analysis of the confocal videos, using Imaris software, allowed us to follow the morphology and behaviour of the cells in three dimensions over time (supplementary Video S9). This level of analysis, not only allowed us to assess the changes in morphology of the cells over time, but also the movement of the internalized bacteria within the intracellular compartments as the cells moved (supplementary Video S9). In summary, these data show that C/EBPα is sufficient to precociously trigger an entire myelopoiesis pathway in naïve pluripotent prospective ectodermal cells resulting in terminally differentiated and fully functional primitive myeloid cells.
Discussion

While much is known about the molecular and cellular pathways that underlie definitive myelopoiesis, relatively little is known about the mechanisms responsible for primitive myelopoiesis. In the mouse, the first hematopoietic cells arise from the blood islands in the yolk sac, soon after the completion of gastrulation.\(^{37}\) While the main cell type that arises from the yolk sac is primitive erythroblasts, the yolk sac also contains a small population of mature macrophages, arising from the mother and from the yolk-sac proper.\(^{38}\) While the ontogeny of the yolk sac derived primitive myeloid cells has been investigated in the mammalian embryo, very little is known about the molecular events that lead their specification.\(^{39}\) Given the relative difficulty in studying this small population of cells in mammalian embryos, we have begun to study the molecular events that lead to the specification of primitive myeloid lineages in amphibian embryos.\(^{15}\)

We have previously shown that primitive myeloid cells are the first blood cells to differentiate and become functional in the *Xenopus* embryo.\(^{15}\) Here we show that *cebpa* is one of the earliest genes to be activated in the hematopoietic pathway. Furthermore, we show that *cebpa* function is necessary for proper primitive myeloid cell differentiation. However, strikingly, we found that overexpression of C/EBP\(\alpha\) was sufficient to initiate primitive myeloid development in pluripotent embryonic cells. Interestingly C/EBP\(\alpha\) initiated hematopoiesis without the cells transitioning through a general early mesodermal state, suggesting that C/EBP\(\alpha\) can bypass the general mesodermal state and initiate myelopoiesis directly from pluripotent embryonic cells. Importantly, we found that C/EBP\(\alpha\) was not only sufficient to induce a precocious expression of several hemotopoietic and myeloid genes, but C/EBP\(\alpha\) was
singularly capable of driving an entire primitive myelopoiesis pathway resulting in functional myeloid cells. To date, no other single factor has been described which is able to drive an entire myelopoiesis pathway in naïve pluripotent cells.

While overexpression of C/EBPα can induce precocious primitive myeloid development in pluripotent embryonic cells in *Xenopus*, knocking down *cebpa* in embryos did not completely prevent the initial specification of endogenous primitive myeloid progenitors. For example, *spib*, a gene known to be important for the specification of primitive myeloid cells, was not affected by knocking down *cebpa*, although overexpression of *cebpa* can potently induce this gene. Similarly, hemangioblast markers, *scl* and *fli1*, were not affected in the *cebpa* morphants but were induced by *cebpa* in animal caps and in embryos. These data suggest that, while *cebpa* is dispensable for the early specification of primitive myeloid progenitors, it can singularly initiate primitive myelopoiesis. There are several possible reasons for this apparent discrepancy. One possible reason is redundancy; experiments in mammals have suggested that members of the C/EBP family can functionally compensate for each other. For example, replacing C/EBPβ into the C/EBPα locus can completely restore hematopoiesis. Indeed, *cebpb* and other members of the C/EBP family appear to be expressed in early *Xenopus tropicalis* embryos, as suggested by the presence of ESTs for these genes in early embryonic cDNA libraries (http://informatics.gurdon.cam.ac.uk/online/xt-fl-db.html). Thus, *cebpb* or other C/EPBs could be acting in a partially redundant fashion with *cebpa*. Secondly, we have noted that, although *cebpa* is expressed in the anterior mesendoderm in gastrula embryos, this gene is also expressed maternally. Therefore, there could be some compensation in the knock down embryos from maternally provided protein. Finally, while *cebpa* can singularly initiate primitive myelopoiesis
in the animal cap, in the embryo cebpα may normally act in combination with other factors to promote hematopoeisis. It will be important in the future to determine which other factors function in combination with C/EBPα to drive primitive myelopoiesis in the embryo.

Currently a large amount of effort has been placed towards identifying factors that can drive pluripotent embryonic stem (ES) cells towards specific developmental fates. Recently C/EBPα has been shown to be a potent factor, which can induce either transdifferentiation or dedifferentiation depending on the experimental regime. For example, forced expression of C/EBPα in lymphoid cells can induce transdifferentiation into myeloid cells in a Pu.1 dependent manner.42,43 Furthermore, C/EBPα, in combination with Pu.1, is able to convert fibroblasts into macrophage-like cells.44 Finally, C/EBPα was recently shown to help reprogram terminally differentiated B cells toward a pluripotent stem cell state in combination with Oct4, Sox2, Klf4 and c-Myc.45 Here we extend these findings by showing that C/EBPα alone can initiate and drive an entire primitive myelopoiesis program in pluripotent embryonic cells in Xenopus. In future studies, it will be important to determine whether C/EBPα can induce myelopoiesis in human or mouse embryonic stem (ES) cells. After all, when designing strategies to generate defined cell types for therapeutic purposes, single factors, such as C/EBPα, which can initiate an entire developmental program from pluripotent cells is of particular interest.
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Authorship

Contributions: Y.C. performed and analyzed most of the experiments, prepared the figures and co-wrote the manuscript; R.M.B.C. and M.R. cloned and performed the initial expression analysis on cebpa. R.M.B.C also assisted in the animal cap transplants, the phagocytosis experiments and confocal imaging. N.L. and X.S. assisted in the real-time PCR analyses. R.P. identified the putative C/EBP binding sites in the spib and lurp promoters. E.A. assisted in the animal cap transplantations, guided the project and co-wrote the manuscript.

Conflict of interest disclosure: The authors declare no competing financial interest.

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FIGURE LEGENDS

Figure 1. Expression pattern of hematopoietic genes in *Xenopus tropicalis*. Whole-mount *in situ* hybridization analysis of *cebpa, spib, scl, fli1, mpo, csfr1, spi1, lcp, mmp7* and *globin* at Stage 12, 15, 18, 23 and 26. Expression of *cebpa* was first detected at Stage 12 in dorsal anterior mesoderm (also see Fig. S1, in supplementary material). From stage 15 until the end of neurulation, *cebpa* was expressed in the anterior ventral blood islands (aVBI). Shortly after, *cebpa* positive cells showed a punctate pattern throughout the embryo. A: anterior view; D: dorsal view; L: lateral view, anterior is to the left, and dorsal is to the top; V: ventral view, anterior is to the left.

Figure 2. *cebpa* loss-of-function phenotype. *Xenopus tropicalis* embryos injected with control MO (10ng; A, C, E, G, I, K) or *cebpa*ATG MO (10ng; B, D, F, H, J, L), fixed at stage 23 and analyzed by whole mount *in situ* hybridization for the myeloid markers *mpo* (A, B), *lcp* (C, D), *mmp7* (E, F), *spi1* (G, H), *spib* (I, J) and *cebpa* (K, L). All images are lateral views, anterior to the left. The ratio of embryos showing the phenotype is shown in the bottom right corner of each image.

Figure 3. HA-*cebpa* mRNA rescued *cebpa* loss-of-function phenotype. (A) *Xenopus tropicalis* embryos injected with *cebpa*ATG or control MO at 1-cell stage (i) were allowed to develop to the 32-cell stage (ii), and then co-injected with HA-*Cebpa* mRNA or eGFP mRNA and β-gal tracer mRNA into the two C1 blastomeres, which are fated to give rise to the aVBI. The embryos were fixed at stage 23 and stained for X-gal (iii-v) to assess the migration of the
injected cells. (B) The extent of migration of X-gal positive cells was scored by placing a grid over the embryos and determining the percentage of the total area of each embryo, that contained X-gal positive cells. Error bars represent standard error mean (s.e.m.) of ten embryos. Statistical ANOVA analysis was done using the SPSS software package. Compared to the control MO injected embryos, the cebpaATG injected morphants showed a significantly reduced extent of migration (P<0.001), which was rescued by HA-cebpa mRNA to a level similar to that seen in the control embryos (P=0.678). (C) Real-time PCR analysis on embryos at stage 23 injected with cebpaATG or control MO, co-injected with HA-cebpa mRNA or eGFP mRNA. Primitive myeloid markers mpo, lcp, mmp7 and spi1 were significantly reduced by cebpaATG MO (i-iv) and were rescued to normal or even higher levels by HA-C/EBPα. Expression levels were normalized relative to rpl8. Error bars represent standard error mean (s.e.m.) of four independent experiments. Statistical ANOVA analysis was done using the SPSS software package. The experimental conditions that showed a significant difference in expression level relative to the control MO + gfp mRNA injected embryos are labeled as *: P<0.05; **: P<0.01; ***: P<0.001.

**Figure 4. cebpa gain-of-function phenotype.** Overexpression of cebpa induced ectopic expression of blood markers on *Xenopus tropicalis* embryos. Embryos randomly injected with 10 pg cebpa mRNA into one cell at 8-cell stage were fixed at stage 18 for whole mount in situ hybridization for spib (A-D) and mpo (E-H). Panels A, B, E and F are dorsal views, with anterior to the left. Panels C, D, G and H are lateral views, with anterior to the left and dorsal to the top.
Figure 5. cebpa induces blood markers in animal caps. (A) Injection of cebpa mRNA in the animal pole at 1-cell-stage induced precocious expression of all blood markers, with the exception of globin and rag1, in Xenopus laevis animal caps. eGFP mRNA was injected as control. Animal caps were excised at stage 8, and cultured in 0.2% BSA, 75% NAM until their sibling whole embryos reached stage 11 or stage 15. At stage 11, spib (i), lurp (ii) and fli1 (v) were strongly up-regulated while mpo (iii), mmp7 (iv) and scl (vi) were strongly up-regulated at stage 15. In contrast, globin (x), rag1 (xi), and several non-hematopoietic markers, including gsc (vii), xbra (viii) and keratin (ix), were not induced. Ø: undetectable expression level. (B) The early-induced genes, spib and lurp, were activated in the presence of CHX (i, ii), while the late induced genes mpo and mmp7 were not (iii, iv). Expression levels were normalized relative to rpl8. Error bars represent standard error mean of three independent experiments. Statistical ANOVA analysis was done using the SPSS software package. Conditions which showed a significant difference in expression level when comparing the cebpa overexpressing animals caps with and without CHX are labeled *: P<0.01; **: P<0.001.

Figure 6. cebpa does not induce mesodermal markers in animal caps. (A) One-cell stage embryos were injected with either gfp or cebpa mRNA. Animal caps were excised and RNA was collected when sibling embryos reached stage 8, 9, 10 and 11. Real-time PCR showed that xbra (i), gsc (ii) and eomes (iii) were highly up-regulated during gastrulation stage in the whole embryo (stage 8-11). However, these mesodermal marker genes were not induced in neither the gfp nor the cebpa mRNA injected animal caps (i-iii). In contrast, the myeloid marker spib (iv) was rapidly up-regulated in cebpa injected animal caps but remained undetectable in either gfp
injected animal caps or whole embryos. (B) One-cell stage embryos were injected with either gfp or cebpa mRNA, and treated with DMSO or a combination of two inhibitors (SU5402 and SB505124), which inhibit the FGF and nodal receptors, respectively, from the 2-cell stage onwards. RNA was isolated from whole embryos and animal caps at stage 11. Real-time PCR analysis showed that cebpa induced the expression of the myeloid markers, spib (iii), lurp (iv) and mmp7 (v), even when the combination of FGF and nodal inhibitors inhibited the expression of the early mesodermal genes, xbra (i) and gsc (ii). Expression levels and ΔCt values were normalized relative to rpl8. Error bars represent standard error mean of three independent experiments.

**Figure 7. Ectopic expression of C/EBPα induces myelopoiesis programme in pluripotent embryonic cells.** (A) Experimental setup: stage 8 animal caps were transplanted from cebpa and/or eGFP mRNA injected embryos to unlabelled host embryos. Chimeras were recorded for live imaging (see Videos 1-9 in supplementary material). (B) The boundaries between the eGFP positive transplants and non-fluorescent host embryos remain clear throughout embryonic development in the control eGFP transplants (i, ii). In contrast, blurred transplant margins and spotty pattern of eGFP positive cells were observed in the embryos containing cebpa expressing transplant cells (iii, iv). Confocal imaging at stage 40 showed that a subset of these scattered cells showed ramified morphology, reminiscent of macrophages (v). (C) Migration route of cebpa induced primitive myeloid cells before and after wounding. At stage 34, C/EBPα(+) graft derived migratory cells patrolled randomly before wounding (i, also see Video 4 in supplementary material). Note that only a subset of GFP/CEBPα cells are migratory, while
others retain the morphology of epidermal cells. After wounding, migratory GFP/CEBPα cells were immediately recruited to the wound site (ii, also see Video 5, in supplementary material). Panels i and ii show the migratory paths of the cells before (i) and after (ii) wounding, as traced manually in Videos 4 and 5, respectively. Different colors were used to distinguish the migration routes of different cells. (D) Three-dimensional xyz confocal sections (20x Objective, 1.0x zoom) of a macrophage (eGFP, green channel) which has phagocytosed bacteria (mCherry in red). Red bacteria are present in intracellular compartments surrounded by cytoplasm in the macrophages (green). For Panel B (i-iv) and C, images were obtained on a fluorescence stereoscope Leica MZ FLIII (Wetzlar, Germany) attached to a Sony CCD camera DXC-950 image capture system controlled by Northern Eclipse Software 7.0 (Empix Image, Mississauga, ON); 0.1×MMR was used as imaging medium. For Panel B (v) and D, images were taken under Olympus IX81 FluoView FV1000 confocal microscope (Olympus Inc., Tokyo, Japan); 0.1xMMR, 2% Methylcellulose (Sigma) 0.01% MS222 was used as imaging medium.
Figure 2

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- **mpo (A)**: Control MO shows a pattern with 28/31 positive embryos.
- **lcp (C)**: Control MO shows a pattern with 14/14 positive embryos.
- **mmp7 (E)**: Control MO shows a pattern with 25/25 positive embryos.
- **cebpATG (B)**: Shows a pattern with 11/13 positive embryos.
- **spi1 (G)**: Control MO shows a pattern with 16/17 positive embryos.
- **spib (I)**: Shows a pattern with 32/33 positive embryos.
- **cebpApa (K)**: Shows a pattern with 25/28 positive embryos.
- **cebpATG (H)**: Shows a pattern with 10/14 positive embryos.
- **cebpATG (J)**: Shows a pattern with 19/23 positive embryos.
- **cebpATG (L)**: Shows a pattern with 16/20 positive embryos.

Figure 3

A

1-cell stage

32-cell stage

i

cebpA MO or control MO

HA-cebpA or gfp + β-gal

cell MO+gfp mRNA

cebpA MO+HA-cebpA mRNA

stage 23

B

p=0.678

p<0.001

scored migration %

stage 23

control MO + gfp mRNA

cebpA MO + gfp mRNA

cebpA MO + HA-cebpA mRNA

C

normalized expression

i

mpo

lcp

mmp7

spi1

spib

control MO + gfp mRNA

cebpA MO + gfp mRNA

control MO + HA-cebpA mRNA

cebpA MO + HA-cebpA mRNA
Figure 5

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Legend:

- 1 gfp whole
- 2 gfp caps
- 3 cebpapa caps

Normalized Expression

CHX - +

- - +
C/EBPα initiates primitive myelopoiesis in pluripotent embryonic cells

Yaoyao Chen, Ricardo M.B. Costa, Nick R. Love, Ximena Soto, Martin Roth, Roberto Paredes and Enrique Amaya

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